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Solubilities of the Derivatives of β-Lactoglobulins A and B Produced by the Action of Carboxypeptidase A

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The solubilities of the derivatives of β-lactoglobulins A and B prepared by the action of carboxypeptidase A were determined in water and sodium chloride solution. It was found that solubility of both A and B β-lactoglobulin decreased rapidly with the removal of C-terminal isoleucine and penultimate histidine, both forms having essentially the same solubility in water and sodium chloride solutions after the removal of 2 moles each of these amino acids per mole of protein. The finding that the difference in solubility of β-lactoglobulins A and B is eliminated by the removal of the same amino acids from the carboxy terminal chain suggests an interaction between the genetic variant amino acid residue and the C-terminal amino acid, which produces a rather large effect on the solubility of the protein.

Previous solubility studies on the genetic variants of β-lactoglobulin have shown that type B, containing 2 more alanine and glycine and 2 less valine and aspartic acid residues than type A, is much more soluble in water and dilute salt solutions than type A (1–3). If, however, the solubility in sodium chloride is divided by the solubility in water, the logarithm of the ratio is the same for any given salt concentration for both type A and B β-lactoglobulins, indicating the same number of dipoles in these two types of β-lactoglobulin (3). It became of interest to determine if these solubility relations of β-lactoglobulin would also be true of β-lactoglobulin derivatives produced by the action of carboxypeptidase A in which the C-terminal end groups are removed as described by Davie *et al.* (4) and also by Kalan *et al.* (5).

The present paper describes the solubilities of these crystalline derivatives of β-lactoglobulins A and B produced by the action of carboxypeptidase A.

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MATERIALS AND METHODS

Preparation of β-lactoglobulin. Milk was obtained from individual Jersey cows the β-lactoglobulin of which had been previously typed by gel electrophoresis. Milk from two cows was combined for the preparation of β-lactoglobulin A.

The method of Palmer as modified by Treece *et al.* (3) was used to prepare β-lactoglobulin. The products were crystallized 4 times by dissolving in 0.1 *M* NaCl and dialyzing against distilled water and stored at 5° under toluene.

Preparation of β-lactoglobulin derivatives. The methods described by Davie *et al.* (4) and Kalan *et al.* (5), with some modification, were used to prepare the β-lactoglobulin derivatives. A commercial 3×-crystallized preparation of carboxypeptidase A, treated with diisopropylfluorophosphate (DFP), was used in water suspension. β-Lactoglobulin was dissolved by adding 0.1 *N* NH_4OH to an aqueous suspension of the crystals until the pH reached 7.9. Any insoluble material was removed by centrifugation. The β-lactoglobulin solution was diluted to 250 ml and a protein concentration of 2–3% as determined by absorption at 280 mμ using an extinction coefficient of 9.7. Fifty mg of carboxypeptidase A were added and kept in suspension by stirring. The enzyme was allowed to react for 3 hours at 37°. Changes in the optical rotation were followed during the incubation period. The carboxypeptidase was then re-

TABLE I

CONTENT OF SELECTED AMINO ACIDS IN β-LACTOGLOBULINS A AND B AND IN THEIR DERIVATIVES PRODUCED BY CARBOXYPEPTIDASE A

Amino acid	Residues per mole of protein[a]									
	βLg		βLg, 3 hour derivative				βLg, 6 hour derivative			
	A	B	A		B		A		B	
			No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2
Glycine	6.1	8.2	6.1	6.0	8.0	7.9	6.1	6.1	8.0	8.1
Aspartic acid	32.4	30.4	32.8	31.8	30.6	29.9	32.2	32.8	30.7	30.6
Alanine	28.0	30.1	28.4	27.9	30.1	28.7	28.0	27.8	30.6	30.1
Valine	19.3	17.5	20.0	19.5	18.2	17.5	19.4	19.3	17.8	17.9
Isoleucine	19.4[b]	19.5[b]	17.4	17.3	17.3	16.5	17.0	18.5	17.4	17.5
Histidine	3.7	3.8	2.4	2.2	3.1	3.0	1.9	1.9	2.2	2.3

[a] Calculated on the basis of 8 residues of phenylalanine per mole of protein and on analysis of 24- and 72-hour hydrolyzates.

[b] Value from Gordon *et al.* (12).

moved by centrifugation and the pH of the supernatant adjusted to pH 5.5 by the addition of 0.5 *N* HCl. Crystals of the derivative formed immediately and, after standing for 1 hour, were removed by centrifugation. After washing with water, the crystals were dissolved in 0.2 *M* NaCl. A small amount of insoluble material was removed and the crystalline derivative was obtained by dialysis of the supernatant. The product was recrystallized 4 times in this manner and stored under water and toluene at 5°.

The amino acid content and solubility of the derivatives prepared by a single 3-hour period of hydrolysis with carboxypeptidase were determined. However, the histidine content was greater than the value consistent with the removal of two histidine residues per mole of protein. Consequently, the derivatives were dissolved in NH_4OH and reacted with carboxypeptidase A for a second 3-hour period following the same procedure as was used in the first period of hydrolysis. The solubility of the crystalline derivative produced by the second treatment with carboxypeptidase was so small that it was found to be desirable to dissolve the product in 0.2 *M* $(NH_4)_2SO_4$ instead of 0.2 *M* NaCl in recrystallizing it. A yield of approximately 3 gm of 4×-crystallized β-lactoglobulin derivative which was obtained from 5 gm of β-lactoglobulin after the first 3-hour period of hydrolysis with carboxypeptidase A, was reduced to 2.4 gm after the second 3-hour period of hydrolysis.

Electrophoresis. The purity and type of the β-lactoglobulins were repeatedly verified using the polyacrylamide gel electrophoretic method at pH 8.9 as described by Peterson (6). No differences in the mobilities at pH 8.9 could be detected between β-lactoglobulins A and B and their respective derivatives. Since histidine is essentially uncharged at this pH, its removal should not affect the mobility of the protein.

Amino acid content of the β-lactoglobulin derivatives. The amino acid content of the β-lactoglobulins and their derivatives were determined by the Spackman *et al.* method (7) using an automatic analyzer. The content of variable amino acids in β-lactoglobulins A and B and their derivatives are given in Table I. The remainder of the amino acid composition of these products were all within the range commonly accepted for β-lactoglobulins A and B, illustrating that the hydrolysis by carboxypeptidase removed only C-terminal isoleucine and penultimate histidine. The results recorded in Table I show that 2 residues of isoleucine are removed from both types of β-lactoglobulin in the derivatives prepared by one 3-hour and two 3-hour periods (referred to as 3- and 6-hour derivatives in Table I and subsequently). The reduction of the histidine content of the 3-hour derivatives is less than 2 residues per mole of protein; however, the second treatment with carboxypeptidase further reduced the histidine content. The results as given in Table I indicate that 2 residues of histidine were removed from the type A and slightly less than 2 residues from type B.

Solubility determination. Solubility was determined as previously described (3). A fairly large excess of protein was used in order to avoid possible changes in solubility due to low ratios of protein to solvent similar to that found in β-lactoglobulin. The effect of varying the ratio of protein to solvent was not determined because of the very small solubilities of the β-lactoglobulin derivatives. Solubilities were calculated from absorption

TABLE II

SOLUBILITY OF β-LACTOGLOBULIN DERIVATIVES IN SODIUM CHLORIDE SOLUTIONS AT 25°

Conc. NaCl (*M*)	Solubility G/1000 cc.								Log. Solubility (average)				Log. $\left(\frac{\text{Sol. (salt)}}{\text{Sol. (H}_2\text{O)}}\right)$			
	βLg, 3-hour derivative				βLg, 6-hour derivative				βLg, 3 hour derivative		βLg, 6 hour derivative		βLg, 3-hour derivative		βLg, 6-hour derivative	
	A		B		A		B									
	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	No. 1	No. 2	A	B	A	B	A	B	A	B
0.00		0.28		0.37	0.05	0.05	0.06	0.05	$\bar{1}.41$	$\bar{1}.58$	$\bar{2}.72$	$\bar{2}.74$	0	0	0	0
0.004	0.33	0.38	0.54	—	—	—	—	0.08	$\bar{1}.55$	$\bar{1}.73$	—	—	0.14	0.15	—	—
0.0063	0.43	0.44	0.66	0.69	0.08	0.08	0.09	0.10	$\bar{1}.64$	$\bar{1}.83$	$\bar{2}.90$	$\bar{2}.97$	0.23	0.25	0.18	0.23
0.0125	0.68	0.66	1.08	1.30	0.14	0.14	0.14	0.19	$\bar{1}.83$	0.08	$\bar{1}.15$	$\bar{1}.22$	0.41	0.50	0.44	0.48
0.025	1.57	1.48	1.96	2.15	0.35	0.35	0.31	0.36	0.18	0.32	$\bar{1}.54$	$\bar{1}.53$	0.77	0.71	0.83	0.79
0.050						1.23		1.29			0.09	0.11			1.38	1.37

measurements at 278 mμ using an extinction coefficient of $E^{1\%}_{1cm} = 9.7$.

RESULTS

Solubilities of the β-lactoglobulin derivatives in water and dilute sodium chloride are

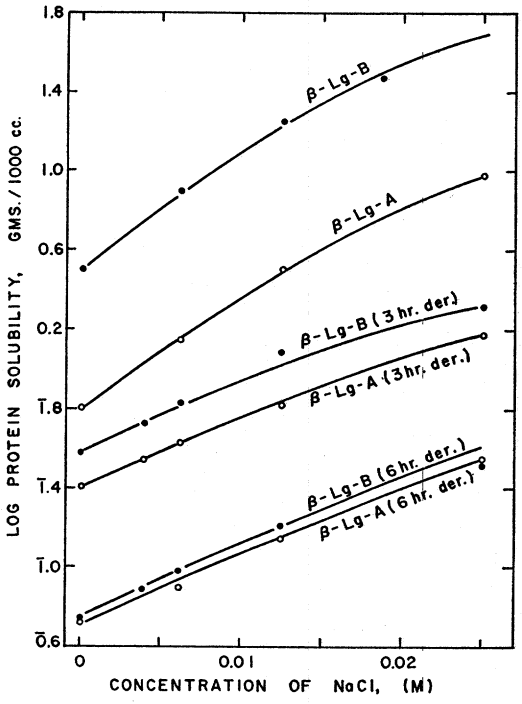

FIG. 1. Solubilities of β-lactoglobulins and β-lactoglobulin derivatives plotted as a function of sodium chloride concentration.

given in Table II and Fig. 1. The reduction in solubility of β-lactoglobulin by the treatment with carboxypeptidase is large in both water and sodium chloride, in agreement with the qualitative observations of Davie *et al.* and Greenberg and Kalan (8). The solubilities of the modified β-lactoglobulins were reduced with each treatment with carboxypeptidase; however, the solubility of the B type was reduced to a much greater extent than that of the A type with the result that the 6-hour B derivative had about the same solubility as the 6-hour A derivative.

The reduction in the solubility of the β-lactoglobulin by treatment with carboxypeptidase was relatively greater in sodium chloride than in water, as illustrated in Fig. 2, as the logarithm of the ratio of the solubility in salt to the solubility in water is considerably smaller for the derivative than the unmodified protein. The solubilities of the derivatives in water are very small and tend to increase with time, perhaps due to an increase in the pH of the solution to values higher than the iso-electric value of 5.2. The solubility values of the β-lactoglobulin derivatives in sodium chloride fall on a straight line when plotted against the square root of the ionic strength but extrapolate to much lower values for the solubility in water than the observed values, similar to the previously reported results for β-lactoglobulin (3). The solubility values in salt, when plotted against ionic strength, however, extrapolate reasonably well to the observed solubility in water, as shown in Fig. 1.

DISCUSSION

The fact that the solubilities of the derivatives of the β-lactoglobulin A and B produced by the action of carboxypeptidase are approximately the same in both water and sodium chloride solutions indicates that the great difference in solubility of unmodified β-lactoglobulin A and B is due to a unique relation between the genetically variable amino acids and the carboxyl end of the chain. Previous investigators have suggested that both the C-terminal and the genetic variant amino acids are near the surface of the molecule. Thus, the finding that the removal of C-terminal isoleucine and histidine produced little change in optical properties and conformation suggested to Greenberg and Kalan (8) that these residues are located on the external surface of the molecule. Watson and Kendrew (9) have also concluded that in nearly all cases of genetic variants of normal hemoglobin the substituted amino acid residues stick outward from the molecule so that substitution would not be expected to affect the tertiary structure. The finding that the A and B type 6-hour derivatives have the same solubility even though the genetic variant amino acids are present indicates that there is some kind of interaction between the C-terminal amino acid and the genetic variant that produces a large effect on solubility of the protein and

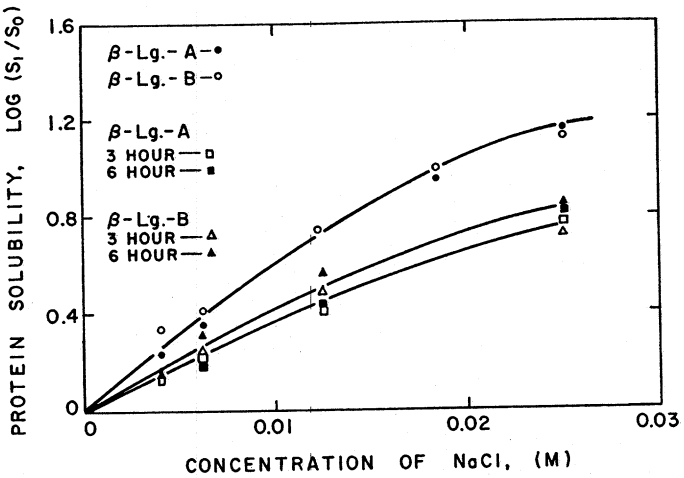

FIG. 2. Solubilities of β-lactoglobulins and β-lactoglobulin derivatives expressed as the log of the ratio of the solubility in sodium chloride divided by the solubility in water as a function of sodium chloride concentration.

that the removal of the C-terminal amino acid destroys the unique solubility effect. It is possible that the hydration of the protein would be affected in a parallel fashion. Experiments on the water content of the crystals of genetic variants and C-terminal modified proteins will be undertaken to determine this point.

Greenberg and Kalan (8) reported that the specific rotations of the carboxypeptidase modified β-lactoglobulins are slightly higher than the unmodified protein. Our results are consistent with this finding since the optical rotation of the β-lactoglobulin solution increased during the action of carboxypeptidase, particularly during the first 3-hour period of hydrolysis and only slightly during the second 3-hour period of hydrolysis, essentially paralleling the rate of removal of amino acids as studied by Kalan and Greenberg (10).

Treece *et al.* (3) reported that when the solubilities of β-lactoglobulins A and B in sodium chloride solutions are divided by the solubility in water, the logarithm of the ratio is the same for each concentration of sodium chloride, as shown in Fig. 2. This finding is consistent with the theory of Scatchard and Kirkwood (11) that the logarithm of the activity coefficient is directly proportional to the ionic strength. The results of this calculation on the solubility values of the β-lactoglobulin derivatives are also shown to follow a similar pattern in Fig. 2, although the variations are larger for the derivatives than for the β-lactoglobulins. This greater variation in log (S_1/S_0) for the derivatives could be due to the difficulties encountered in determining the solubilities in water. The values for log (S_1/S_0) for the 6-hour derivative of β-lactoglobulin B appear to be definitely greater than for the other derivatives; consequently, two curves were drawn for the solubilities of the derivatives. The smaller slopes for the values of log (S_1/S_0) for the β-lactoglobulin derivatives than for β-lactoglobulin indicate that the removal of the two histidine residues reduced the number of dipoles in the molecule.

REFERENCES

1. OGSTON, A. G., AND TOMBS, M. P., *Biochem. J.* **66,** 399 (1957).
2. TOMBS, M. P., *Biochem. J.* **69,** 491 (1958).
3. TREECE, J. M., SHEINSON, R. S., AND MCMEEKIN, T. L., *Arch. Biochem. Biophys.* **108,** 99 (1964).
4. DAVIE, E. W., NEWMAN, CAROLYN R., AND WILCOX, P. E., *J. Biol. Chem.* **234,** 2635 (1959).
5. KALAN, E. B., GREENBERG, RAE, WALTER, M., *Biochemistry* **4,** 991 (1965).
6. PETERSON, R. F., *J. Dairy Sci.* **46,** 1136 (1963).

7. Spackman, D. H., Stein, W. H., and Moore, S., *Anal. Chem.* **30,** 1190 (1958).
8. Greenberg, Rae, and Kalan, E. B., *Biochemistry* **4,** 1660 (1965).
9. Watson, H. C., and Kendrew, J. C., *Nature* **190,** 670 (1961).
10. Kalan, E. B., and Greenberg, Rae, *Arch. Biochem. Biophys.* **95,** 279 (1961).
11. Scatchard, G., and Kirkwood, J. G., *Physik Z.* **33,** 297 (1932).
12. Gordon, W. G., Basch, J. J., and Kalan, E. B., *J. Biol. Chem.* **236,** 2908 (1961).